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Involvement of Novel Multifunction Steroid Hormone Receptor Coactivator, E6-Associated Protein, in Prostate Gland Tumorigenesis

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14. ABSTRACT
E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP), is a novel dual function steroid hormone receptor coactivator. Previously I have shown that E6-AP regulates PI3K-Akt pathway in the prostate gland and as well as LNCaP cells. In this report I have provided evidence that E6-AP plays a vital role in the prostate gland growth and prostate cancer cell proliferation. E6-AP by itself can modulate p53 levels in prostate cancer cells independent of E6. Our data also indicates that over expression of E6-AP could potentially lead to tumor initiation. I have also shown that E6-AP also affects the non-genomic mechanisms of AR. ChIP assays demonstrate that AR is accumulated on its target promoter and enhancer under E6-AP knockdown conditions. Taken together, these studies indicate that E6-AP plays a vital role in both the genomic and non-genomic signaling pathway of AR and their downstream effectors might play critical roles in many biological processes, especially in cell growth.

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Introduction

Even though prostate cancer is known to be the most common malignancy and the second leading cause of cancer death in American males, the molecular basis of the disease and the mechanisms by which it becomes hormone refractory remains unknown. In order to more fully develop effective prevention and intervention strategies for this prevalent disease, the underlying molecular mechanisms of initiation and progression must be understood. The development, growth and maintenance of the prostate gland is androgen dependent and evidences point out that androgen receptor (AR) and its coactivators are important in prostate cancer. Therefore, it will be important to investigate in what capacity the AR coactivators are involved in the development of prostate tumors and in the progression of hormone resistance. In this context genetically engineered mouse models can provide significant advantages for studying the molecular mechanisms of prostate carcinogenesis.

1) Androgen Receptor (AR) and its Coactivators

The effects of hormones or androgens on the development of the normal prostate gland and prostate tumors are mediated through an intracellular receptor called AR (1, 2). In the absence of hormone, AR is located in the cytoplasm of target cells and is associated with cellular chaperones. In order to activate gene transcription, the AR undergoes a series of well-defined steps. When bound to hormone, the AR undergoes a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with coactivators and recruitment of chromatin modifying enzyme activities, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex (PIC). These events are followed by up- or down-regulation of target gene expression (3-6). However, AR may also be converted into an active form even in the absence of androgen (7-9). The mechanism of hormone-independent activation of AR has not been understood fully yet but it may involve the bypassing of any one of the above mentioned steps of hormone-dependent activation.

Coactivators represent a growing class of proteins, which interact with receptors including the AR in a ligand-specific manner and serve to enhance their transcriptional activity (10-14). A number of coactivators have been cloned to date, including SRC-1 family members (15-19), PGCs (20), SRA (21), CBP (22-24) and **E6-associated protein (E6-AP)** (25) etc. and this list is growing rapidly day by day. In addition to these coactivators, a series of other AR coactivators, ARAs, has also been discovered such as ARA160 (26), ARA70 (27), ARA55 (28), ARA54 (28) and ARA24 (29). Coactivators have been shown to possess enzymatic activities, such as histone acetyltransferase, histone methyltransferase, ubiquitin-conjugation, and ubiquitin-protein ligase. Presumably, the coactivator's *in vivo* functions manifest by congregating their enzymatic activities to the promoter region of the target gene which contribute to their ability to enhance receptor-mediated transcription (10). Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological response to steroids, vitamin D, and retinoids in different tissues or from individual to individual.

3) E6-associated Protein (E6-AP) as a Coactivator

Our lab has cloned E6-AP as steroid hormone receptor interacting protein. E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, including that of AR (25). E6-AP was previously identified as a protein of 100 kDa, which mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53 (30). The E6/E6-AP complex specifically interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome

pathway. E6-AP also degrades p53 independent of E6 protein. Recent evidences reveal that protein ubiquitination is a multifunctional signaling mechanism whose regulatory significance is comparable to that of phosphorylation. E6-AP is a member of the E3 class of functionally related ubiquitin-protein ligases (31-33). E3 enzymes have been proposed to play a major role in defining substrate specificity of the ubiquitin-proteasome system. The carboxyl-terminal 350 amino acids (aa) of E6-AP contain a "*hect*" (homologous to the E6-AP carboxy terminus) domain, which is conserved among all E3 ubiquitin protein-ligases and E6-AP related proteins characterized to date. We have shown that the ubiquitin-ligase activity of E6-AP is not required for the coactivation function of E6-AP. This finding indicates that E6-AP possesses two independent, separable functions, coactivation and ubiquitin-protein ligase activity (25).

Contribution of coactivators to prostate cancer development and progression has not been well elucidated. Recently, it has been shown that coactivators SRC-1, TIF-2, and ARA 55 were overexpressed in advanced prostate cancer (34). Overexpression of TIF2 and SRC-1 enhanced AR transactivation at the physiological concentrations of adrenal androgen, suggesting a general mechanism for recurrent prostate cancer growth (35, 36). In other steroidal tumors, it has been shown that altered expression of nuclear receptor coactivator, AIB1, contributes to the development of hormone-dependent breast and ovarian cancer (37). Another coactivator SRA is also elevated in breast tumors (38). We have shown that E6-AP is overexpressed in mouse mammary and prostate tumors (39). These findings suggest that enhanced coactivator expression and activity may aberrantly increase steroid hormone receptor activity and give tumors a selective advantage for proliferation, resulting in the development of aggressive tumor. Considering, the influence of E6-AP as a coactivator on transactivation of target genes by AR and its importance in the development of prostate gland (see preliminary data section), we are interested in studying the role of E6-AP in the development and progression of prostate cancer.

4) PI3K-Akt pathway

In addition to androgen signaling, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway is another important factor that controls the growth and survival of prostate cells. Elevated PI3K-Akt signaling is correlated with prostate cancer progression. It has been suggested that androgen and PI3K/Akt pathways can compensate for each other in growth regulation and prostate development. The PI3K/Akt pathway may up-regulate AR activity by directly phosphorylating AR or through ß-catenin, an AR coactivator. A recent study has shown that the levels of components of PI3K/Akt pathway are elevated and are involved in tumor progression in Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice. Hence, the PI3K-Akt pathway which transduces signals from multiple growth factors and cytokines, apart from regulating cell proliferation, survival and motility, also plays a critical role in modulation of AR activity and prostate cancer.

5) E6-AP knockout mouse line and E6-AP transgenic mouse line

Since E6-AP has been identified as a coactivator of AR in *in-vitro* models, we used E6-AP knockout mice which were generated by targeted gene disruption, to test the effects of loss E6-AP on the normal development of the prostate gland (40, 41). We have also generated E6-AP transgenic mouse line that specifically overexpresses human E6-AP in prostate gland. In this mouse model, E6-AP gene is under the control of rat probasin promoter which specifically targets E6-AP to the prostate epithelial cells. Therefore, these two mice models are excellent tools to study the role of E6-AP in prostate gland development.

Body

In my previous annual report I had hypothesized that E6-AP, a coactivator of AR, is functionally significant in the development of prostate cancer and also plays an important role in PI3K-AKT signaling. Hence, I had earlier proposed the following specific aims:

Aim 1: To study the role of E6-AP in normal prostate gland development, PI3K-Akt signaling and tumorigenisis.
Aim 2: Generation of E6-AP null TRAMP mice and examine the consequence of loss of E6-AP on prostate cancer development and progression in TRAMP mice.
Aim 3: Generation of E6-AP transgenic TRAMP mice and examine the consequence of overexpression of E6-AP on prostate cancer development and progression in TRAMP mice.

However due to technical difficulties encountered to accomplish the earlier proposed aims, we were forced to redirect the aims to focus on the role of E6-AP in regulating signaling pathways other than PI3K-AKT pathway by exploring the non-genomic and genomic mechanisms and targets of E6-AP.

Technical difficulties encountered:

I had earlier reported the difficulties which I encountered in breeding TRAMP mice. During the beginning of the second year of my grant the TRAMP parents gave adequate amount of pups to proceed with the crossing with E6-AP null mice. However, I was forced to drop Aim 2 and 3 and redirect my project towards a new set of aims due to the following reasons.

1. I was not successful in crossing TRAMP with E6-AP null mice
2. There have been reports that TRAMP mice had significant delay or in some cases, a complete absence of generating the cancer phenotype. This has been confirmed by the supplier (Jackson Labs), my peers in our institution and also in my lab.
3. These difficulties have already resulted in significant loss of work hours and have a huge potential to consume all my time on the breeding process which might eventually end up in no significant progress in my project.

As soon as I realized these difficulties, I wanted to pursue my research focusing on E6-AP, with a distinct set of new aims.

Aim 1: To study the role of E6-AP in normal prostate gland development, PI3K-Akt signaling and tumorigenisis.
Aim 2: To study the role of E6-AP in both the genomic and non-genomic mechanisms of AR signaling

I will be summarizing the accomplishments below.

Aim 1: To study the role of E6-AP in normal prostate gland development, PI3K-Akt signaling and tumorigenisis.

In order to study the role of E6-AP in normal prostate gland development, we have created E6-AP transgenic mice. Additionally, we have also created E6-AP over expressing stable cells to study the mechanism by which E6-AP regulates PI3K-Akt signaling. Generation of these cell lines have already been reported in my previous annual report which regulates E6-AP expression (tet-off system) in the presence and absence of doxycycline (dox).

Role of E6-AP in androgen stimulated prostate growth:

Prostate gland growth is androgen dependent and I have previously shown that E6-AP over-expressing transgenic mice are ~20% larger when compared to wild-type littermates. However, to test whether E6-AP plays a role in the ability of prostate gland to respond to androgen signaling, we assessed prostate growth in castrated mice implanted with testosterone slow-release pellets. E6-AP over-expressing transgenic mice and wild-type littermates were castrated for 30 days to deplete the endogenous androgens and testosterone slow release pellets were implanted for additional 21 days. Prostate glands were dissected at 10, 20 and 30 days post-castration and also 21 days after testosterone implantation. Prostate gland involutes after 10, 20 and 30 days post castration (Fig 1), however the rate of involution of E6-AP transgenic mice and wild-type controls remains the same. Also the rate of regeneration of the prostate gland after 21 days of testosterone implantation does not change between E6-AP transgenic mice and wild-type controls. These results indicate that E6-AP has a role in normal prostate gland development, but it does not have any additional effect on prostate gland regeneration under androgen stimulated conditions.

Over expression of E6-AP leads to precancerous lesions:

Initiation and progression of prostate cancer is a multistage process involving a characteristic lesion termed as prostate intraepithelial neoplasia, which is believed to be the precursor for the formation of prostate cancers. Since E6-AP transgenic mice did not give raise to palpable prostate tumors, we decided to do a histological observation of the prostate glands. I have previously reported the development of PIN like structures in E6-AP over-expressing transgenic mice, which were not found in normal wild-type control prostate glands. In this report, I have extended this previous study with additional samples. Prostate glands from >18 month old transgenic and wild-type litter mate controls were dissected into individual lobes (n=10). These tissues were processed, embedded in paraffin and 5μ sections were made. These sectioned tissues were stained for haematoxylin and eosin to determine the morphological features. Figure 2 shows that E6-AP transgenic mice shows hyperplasic or PIN like characteristics when compared with wild-type litter mate controls. This finding suggests that over expression of E6-AP could result in excessive proliferation and formation of preneoplastic lesions which resembles PIN. The results from these studies are tabulated in Table 1.

Overexpression of E6-AP increases the cell growth and proliferation:

The members of the PI3K-Akt signaling pathway have been implicated in cell growth and proliferation. Since, over-expression of E6-AP induces the PI3K-Akt pathway, we predicted that E6-AP stable cell lines will exhibit increased cell growth and proliferation compared to that of untransfected control LNCaP cells. As shown in Figure 2A, E6-AP-LNCaP stable cells that overexpress E6-AP exhibit changes in cell shape compared with control LNCaP cells. E6-AP stable cells lacks cell processes and also clump together. Furthermore, E6-AP stable LNCaP cells show increase in cell size compared to that of control LNCaP cells. In addition to cell shape, we also examined the effects of E6-AP over-expression on prostate cell proliferation using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) growth assays. E6-AP-LNCaP stable cells and untransfected control LNCaP cells were grown for a period of 5 days in media containing normal and as well as charcoal stripped serum. Figure 2B shows a significant difference in proliferation of E6-AP over-expressing stable cells compared with untransfected control LNCaP cells both in normal and stripped serum suggesting that over-expression of E6-AP results in increased cell proliferation both in the presence of normal and stripped serum. These data confirms that over-expression of E6-AP leads to prostate cell proliferation and growth, which correlates with our previous finding that loss of E6-AP leads to reduced prostate gland size and increased apoptosis. Since, PI3K-Akt signaling is elevated in

our E6-AP-LNCaP stable cells and Akt is involved in both hormone-dependent and independent growth of prostate cells, we also examined the role of Akt in prostate cell proliferation. The E6-AP-LNCaP stable cells along with control untransfected parental LNCaP cells were treated with PI3K inhibitor, LY294002 and cell proliferation was examined. Figure 2C shows that PI3K inhibitor, LY294002 significantly inhibit the proliferation of both control LNCaP and E6-AP-LNCaP stable cells in normal serum conditions. The E6-AP-LNCaP stable cells were more susceptible to the inhibitory effects of LY294002. These data suggest that the increase in proliferation observed in E6-AP-LNCaP stable cells is due to increase in the Akt activation.

Role of E6-AP in regulating p53 levels in prostate glands and prostate cancer cells:

E6-AP was initially identified as an E3 ligase which promotes the degradation of p53 during HPV infection in cervical carcinoma cells. However, this effect is E6 dependent, as p53 could only be degraded by the formation of E6 and E6-AP complex. It is not known if E6-AP alone affects p53 degradation in the absence of E6 in prostate cancer cells. To test this, E6-AP over-expressing LNCaP cells were probed for p53 expression using Western blot (Figure 4). LNCaP cells under E6-AP over-expression (-dox) demonstrated a decreased level of p53 compared to normal conditions (+dox), indicating that E6-AP regulates p53 levels independent of E6 in prostate cancer cells. The level of p21 is also downregulated under E6-AP over-expressing conditions, indicating that E6-AP just affects the protein level of p53 and not its transcriptional activity (Figure 4).

Aim 2: To study the role of E6-AP in both the genomic and non-genomic mechanisms of AR signaling

Role of E6-AP in regulating the non-genomic mechanism of AR:

AR regulates many cellular processes by acting as a ligand activated transcription factor resulting in the regulation of the transcription of target genes in the nucleus, referred to as classical mechanism or genomic mechanism. Recent data suggests that AR can also mediate non-transcriptional actions outside the nucleus in addition to its ligand-inducible transcription factor function. These activities of AR are collectively called non-genomic actions which primarily involve rapid activation of signal transduction cascades by phosphorylation (42, 43). For example, androgens, by means of AR, are known to activate PI3K-AKT pathway in a transcription independent manner. In order to study the role of E6-AP in non-genomic actions of AR, E6-AP over-expressing stable cells were tested for the activation AKT at 15 minutes of hormone (DHT) treatment (Figure 5A). DHT-BSA, a membrane non permeable form of DHT was used as a control. Under E6-AP over-expressing conditions the levels of pAKT are elevated compared to normal LNCaP cells. However, the total AKT levels (tAKT) levels remain the same. In addition to modulating rapid activation of pAKT, I also tested the rapid hormone dependent in-vivo interaction of E6-AP with AR. Lysates from both vehicle and short-term DHT treated LNCaP cells were immunoprecipitated with E6-AP specific antibody and then immunobloted with AR antibody. Figure 5B shows that E6-AP interacts with AR within 15 minutes of hormone treatment. These data indicate that E6-AP regulates rapid non-genomic actions of AR, which could be mediated through its rapid hormone specific interaction with AR.

Role of E6-AP in regulating the genomic mechanism of AR:

The genomic mechanism of AR includes binding to the hormone in the cytoplasm, translocation to the nucleus, binding to hormone response elements on the promoter of target genes and regulating gene expression. It also recruits several coactivators which bring in several

enzymatic activities on to the promoter, which aid in gene expression. E6-AP is one such coactivator which has an E3 ligase function, involved in tagging ubiquitin to target proteins for degradation. We have previously shown that E6-AP is involved in degradation of steroid receptors including estrogen receptor and AR. Additonally, we have also shown that E6-AP modulates AR protein levels both in E6-AP null prostate gland and also in E6-AP over-expressing cells. In order to delineate the role of E6-AP on AR genomic actions on target promoters, Chromatin Immunoprecipitation assays (ChIP) were performed to look at AR recruitment on PSA promoter at different time-points of hormone treatment (Figure 6A and B). These ChIP assays were performed under E6-AP knockdown conditions using siRNA against E6-AP (Figure 6C). Interestingly, the recruitment of AR is increased both in the promoter and enhancer regions in the absence of E6-AP at various time points tested (4h, 8h and 16h). These results indicate that in the absence of E6-AP, AR degradation is attenuated leading to accumulation of AR on the promoter.

Key Research Accomplishments

1. E6-AP is involved in the normal growth of the prostate gland; however it does not have any additional affect on the regeneration of prostate gland after castration.

2. Over expression of E6-AP in the prostate gland leads to the development of PIN like lesions

3. Over-expression of E6-AP increases the cell growth and proliferation

4. Over-expression of E6-AP increases non-genomic activity of AR

5. AR is accumulated on its target promoters under E6-AP knockdown conditions

Conclusion

In summary, I have demonstrated that E6-AP, an E3 ligase as well as steroid receptor coactivator, plays a vital role in the prostate gland growth and prostate cancer cell proliferation. I have also provided evidence that E6-AP by itself can modulate p53 levels in prostate cancer cells independent of E6. Our data also indicates that over expression of E6-AP could potentially lead to tumor initiation. I have also shown that E6-AP also affects the non-genomic mechanisms of AR. ChIP assays demonstrate that AR is accumulated on its target promoter and enhancer under E6-AP knockdown conditions. Taken together, these studies indicate that E6-AP plays a vital role in both the genomic and non-genomic signaling pathway of AR and their downstream effectors might play critical roles in many biological processes, especially in cell growth.

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Appendices:

1. Figures

Figure 1

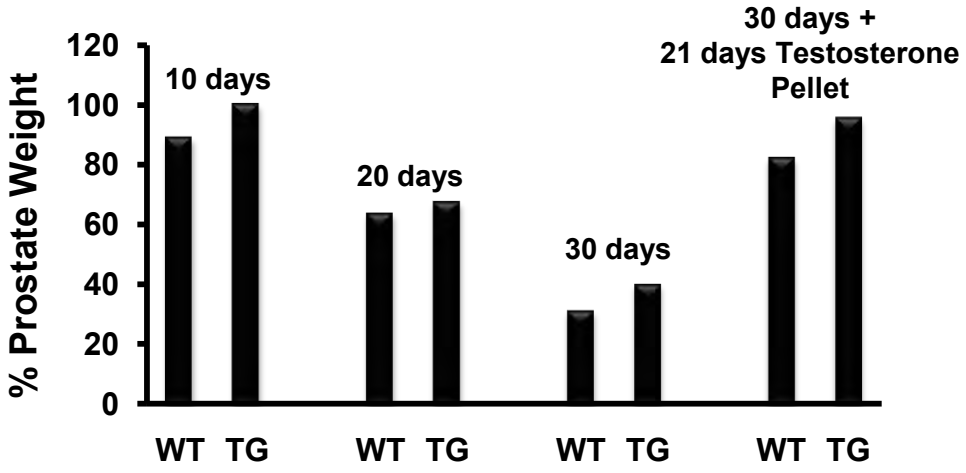


Figure 2

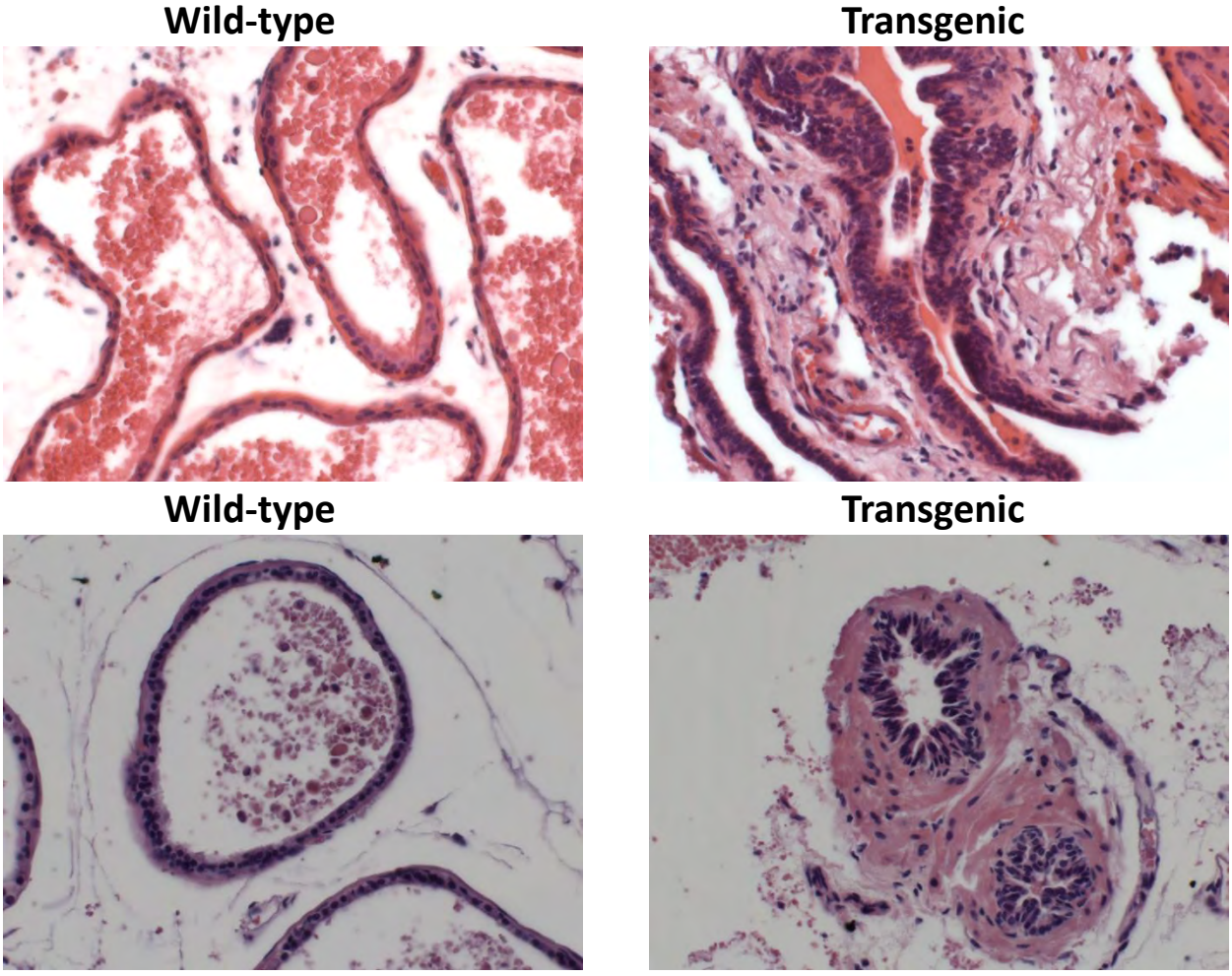
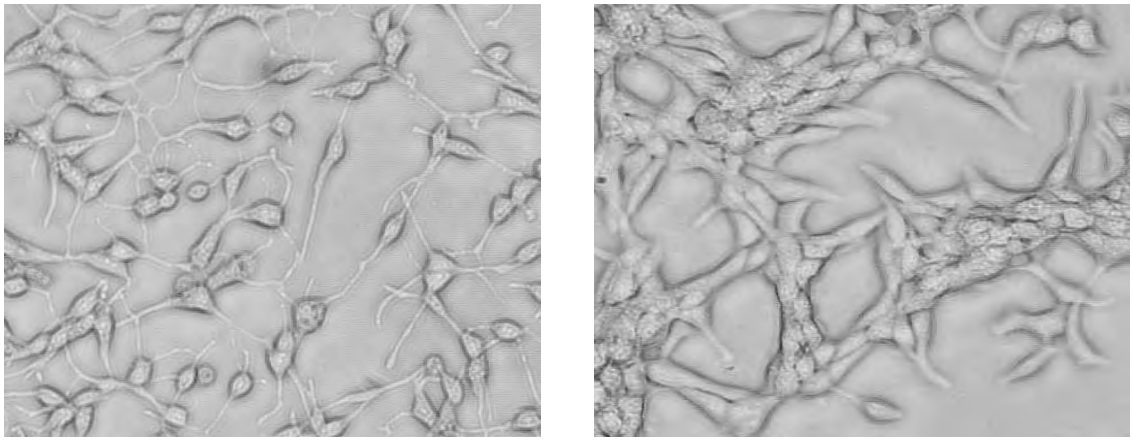


Table 1

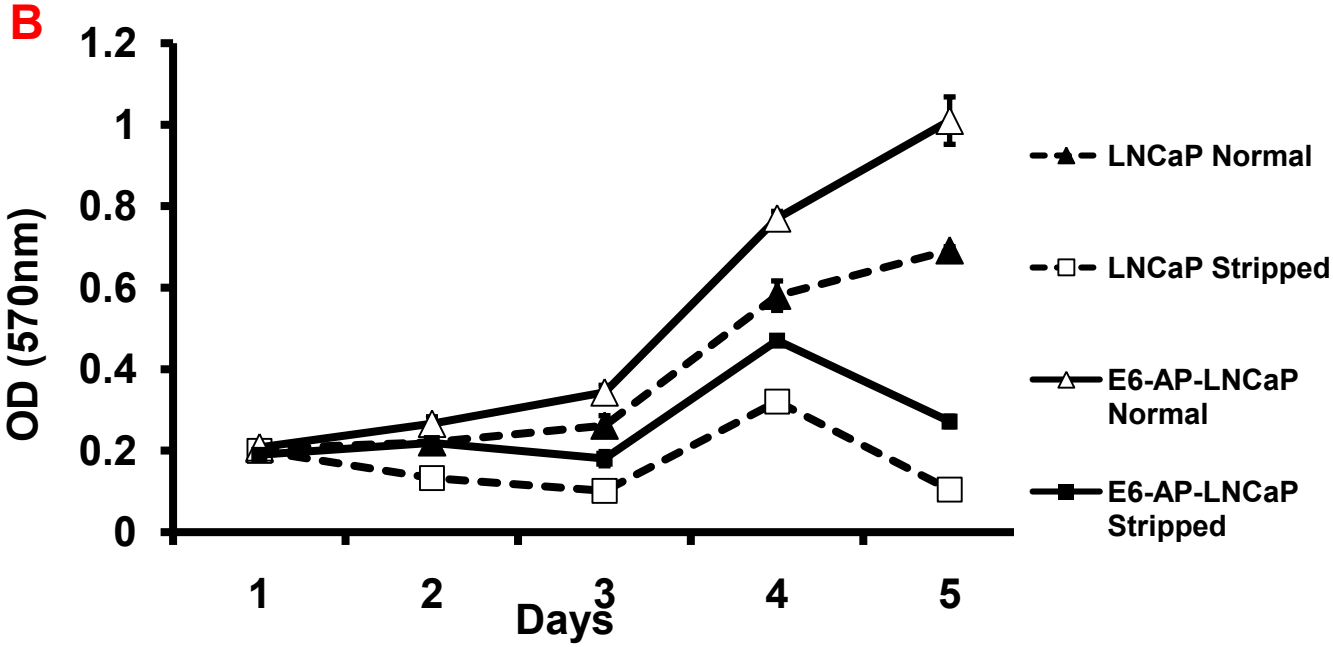
	Total	Normal	PIN
Wild-type	10	10	0
Transgenic	10	4	6

Figure 3

A


LNCaP
E6-AP-LNCaP

B


OD (570nm)
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
Days
LNCaP Normal
LNCaP Stripped
E6-AP-LNCaP Normal
E6-AP-LNCaP Stripped

C

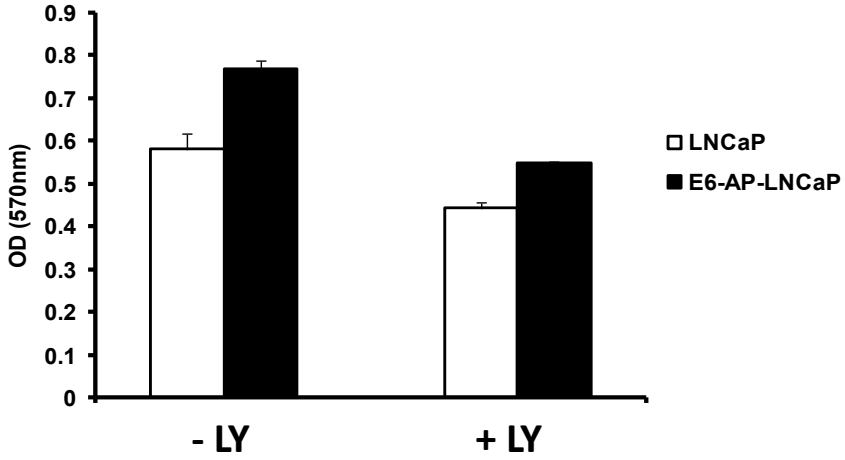

OD (570nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
- LY
+ LY
LNCaP
E6-AP-LNCaP

Figure 4

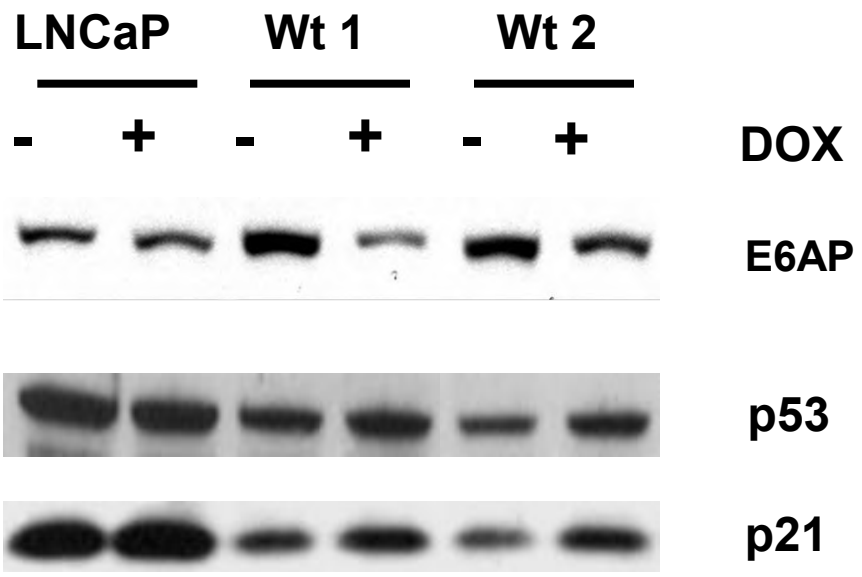


Figure 5

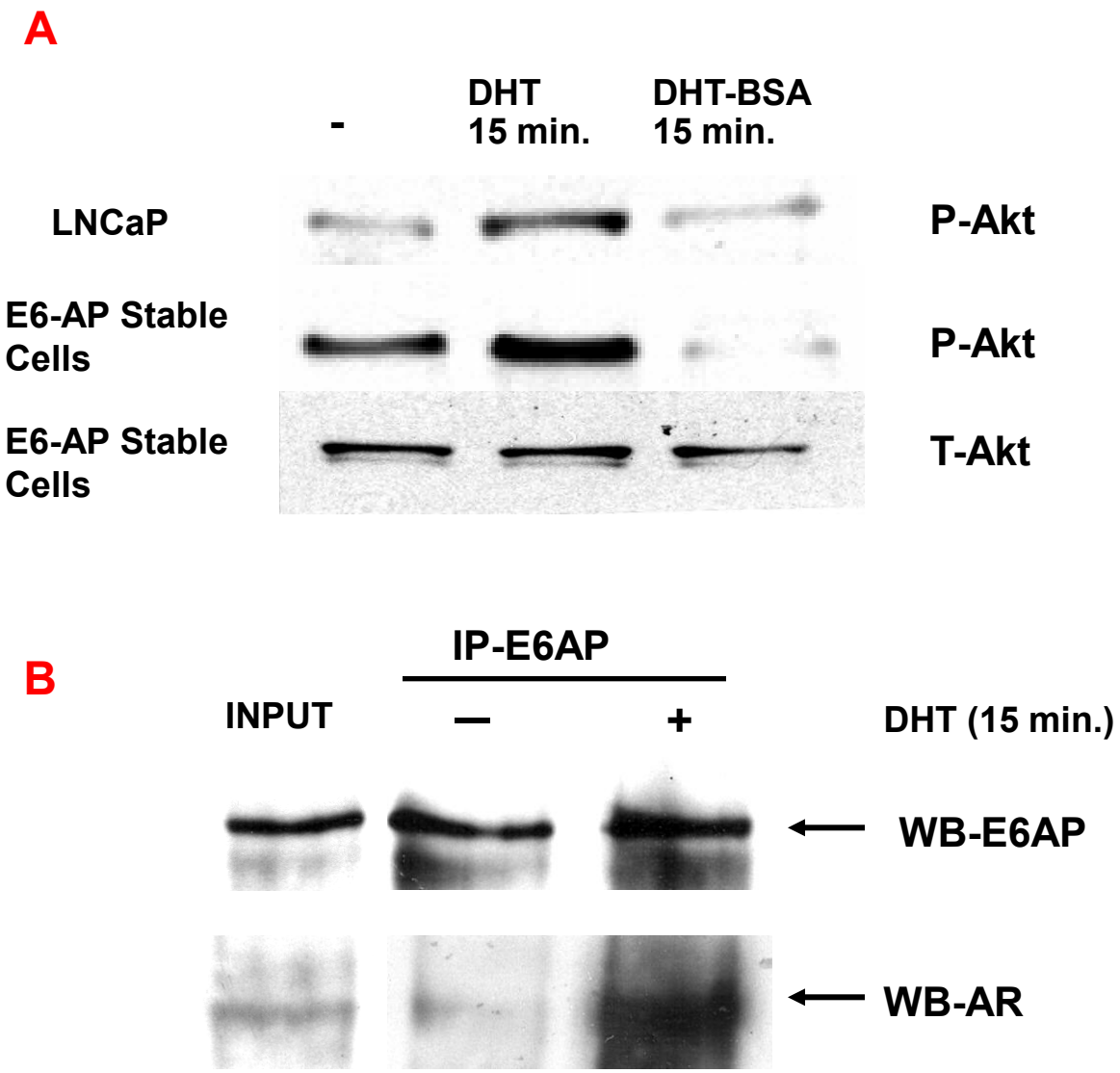
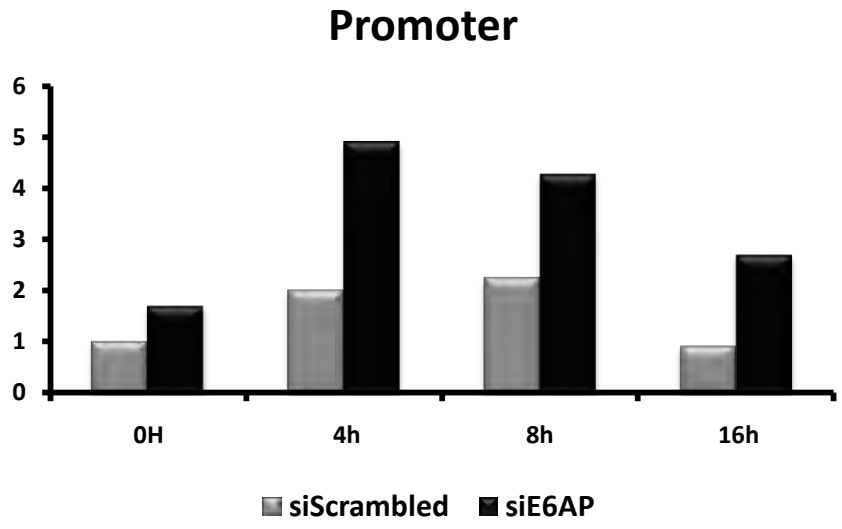
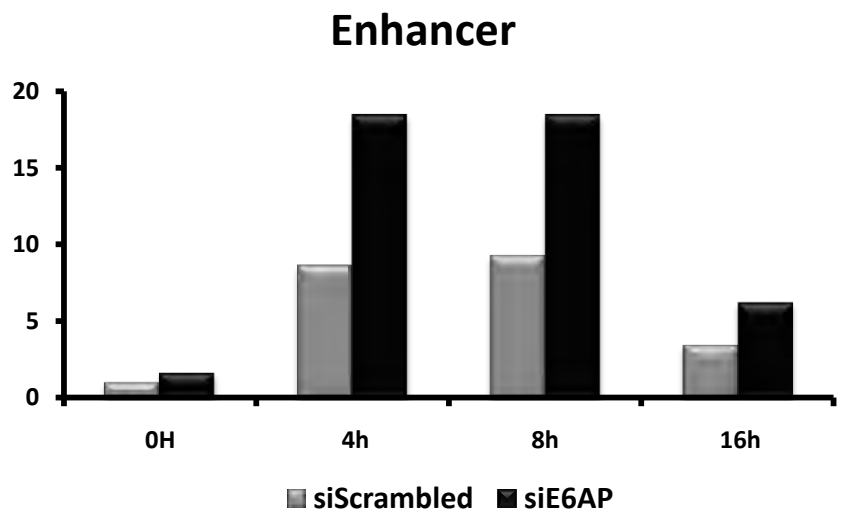

A
-
DHT
15 min.
DHT-BSA
15 min.
LNCaP
P-Akt
E6-AP Stable
Cells
P-Akt
E6-AP Stable
Cells
T-Akt
B
IP-E6AP
INPUT
–
+
DHT (15 min.)
WB-E6AP
WB-AR

Figure 6

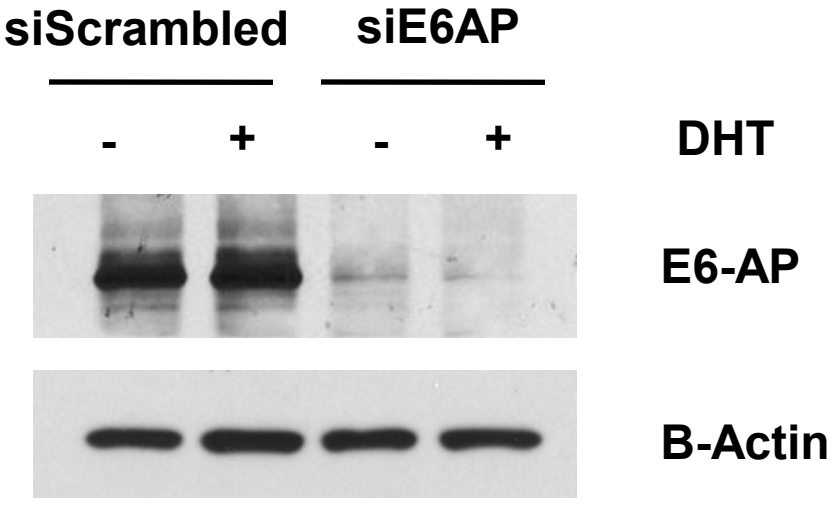
A


Promoter
6
5
4
3
2
1
0
0H
4h
8h
16h
siScrambled
siE6AP

B


Enhancer
20
15
10
5
0
0H
4h
8h
16h
siScrambled
siE6AP

C


siScrambled
siE6AP
-
+
-
+
DHT
E6-AP
B-Actin